João Luís Martins da Gama

Expression of histone modifying enzymes as prognostic biomarkers in follicular lymphoma

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Instituto de Ciências Biomédicas Abel Salazar

Orientador: Rui Manuel Ferreira Henrique

Categoria: Doutor em Ciências Médicas

Diretor do Serviço de Anatomia Patológica do IPOPFG, EPE

Professor Catedrático Convidado do ICBAS-UP

Co-Orientador: Carmen de Lurdes Fonseca Jerónimo

Categoria: Doutor em Ciências Biomédicas

Professor Associado Convidado com Agregação do ICBAS-UP

Investigador Auxiliar e Coordenadora do Grupo de Investigação em Epigenética

e Biologia do Cancro – Centro de Investigação do IPOPFG,EPE IPOPFG,EPE

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Resumo

Introdução: O linfoma folicular é o segundo linfoma Não-Hodgkin mais comum, constituindo cerca de 20% dos casos diagnosticados de novo em adultos e o mais comum dentro dos linfomas indolentes. É reconhecido como um grupo heterogéneo de distúrbios. O *follicular lymphoma international prognostic index* permite a estratificação dos doentes, mas fornece apenas uma estimativa aproximada da evolução da doença. No entanto, têm surgido novos marcadores de prognóstico. Alterações epigenéticas têm vindo a ser demonstradas como sendo uma parte importante e precoce do processo de linfomagénese, contribuindo para a progressão da doença.
Objectivo: Avaliação da expressão das modificações epigenéticas pós-traducionais das histonas H3K4me3 (trimetilação da lisina 4 da histona H3) e H3K27me3 (trimetilação da lisina 27 da histona H3) e correlação com os parâmetros clínicos e patológicos com o objectivo de determinar o seu possível papel como biomarcador de prognóstico em linfoma folicular.
Material e Métodos: Foi realizado um estudo transversal retrospetivo com base em material biológico de arquivo para avaliação da imunoexpressão da marca epigenética H3K4me3 e H3K27me3 (utilizando o Hscore), de 48 doentes do Instituto Português de Oncologia do Porto diagnosticados entre 2007 e 2012. Foi utilizada a base de dados do Serviço de Oncohematologia para obtenção dos dados clínicos e patológicos relevantes.
Resultados: Com base na análise da sobrevivência, o FLIPI ($p<0.001$) e a idade foram confirmados ($p=0.019$) como fatores de prognóstico. Na avaliação das marcas epigenéticas H3K4me3 e H3K27me3, não se observou associação com a sobrevida global.
Conclusões: Nesta série, as marcas epigenéticas H3K4me3 e H3K27me3 não revelaram valor prognóstico em linfoma folicular. Para um melhor estudo será necessário um alargamento do estudo, com um maior número de casos.
Palavras-Chave: Linfoma não Hodgkin, Linfoma folicular, epigenética, biomarcadores moleculares, imunohistoquímica, H3K4me3, H3K27me3, prognóstico

Abstract

Background: Follicular lymphoma is the second most common subtype of non-Hodgkin lymphoma, accounting for approximately 20% of all newly diagnosed cases, and it is the most common indolent non-Hodgkin lymphoma. It is characterized by significant heterogeneity. The follicular lymphoma international prognostic index is used to predict outcome, but gives only a rough estimate. Additional prognostic markers are under investigation. Epigenetic changes are now recognized as playing an important and early role in the lymphomagenesis process, contributing to the disease progression.

Objective: Quantitative evaluation of the expression of the post-translational epigenetic modification of H3K4me3 (trimethylation of lysine 4 on histone H3) and H3K27me3 (trimethylation of lysine 27 of histone 3) in a series of follicular lymphomas through immunoexpression and correlation with clinicopathological parameters, to determine its relevance as prognostic biomarker.

Material and Methods: A retrospective transversal study was completed using archival biological material from 48 patients with follicular lymphoma, diagnosed between 2007 and 2012 at the Portuguese Oncology Institute Porto. The H3K4me3 and H3K27me3 marks were assessed by immunohistochemistry, using the Hscore. Relevant clinical and pathological data was extracted from the database of the Department of Oncohematology.

Results: In this series, only the FLIPI score ($p<0.001$) and age at the time of diagnosis ($p=0.019$) were confirmed as a prognostic factor. When evaluating the epigenetic marks H3K4me3 e H3K27me3 no significant association with clinical outcome was found.

Conclusions: In this study, the immunoexpression H3K4me3 and H3K27me3 didn't show prognostic value. A larger study is needed in order to fully determine the relation between H3K4me3, H3K27me3 and prognosis.

Keywords: Non-Hodgkin lymphoma, follicular lymphoma, epigenetics, molecular biomarkers, immunohistochemistry, H3K4me3, H3K27me3, prognosis

Non-Hodgkin lymphomas (NHL) are a diversified and heterogeneous group of disorders derived from the clonal expansion of B-cells, T-cells, or NK cells.
Among NHL, follicular lymphoma (FL), a low-grade B-cell neoplasm, is the second most common histological subtype and the most frequent indolent NHL [1], accounting for approximately 20% of all NHL cases. It is characterized by significant heterogeneity [2] with regards to clinical presentation, morphological spectrum and overall survival.

FL usually arises in the lymph nodes, it is most often clinically indolent and grows more slowly compared with some other forms of NHL. Although most FLs are at an advanced stage at the time of diagnosis, about 50% of the patients survive at least 8 to 10 years after diagnosis, and many may not require treatment for a long time. There is no known curative therapy, and treatment largely depends on the stage of the disease and, thus, initial staging is paramount [3]. FL patients may be treated with a combination of chemotherapy, monoclonal antibodies and/or radiation therapy, or they may be followed closely with watchful waiting [3]. Treatment of FL is challenging because of frequent recurrence [1].

FL occurs in all races and at all geographic locations, but the exact worldwide incidence is not known. In Europe, the incidence of FL is approximately 2.18 cases per 100.000 people per year [4], whereas in the United States the annual incidence has been estimated as 3.18 per 100.000 people. There is no difference in incidence between sex. The incidence increases with age, it is higher in Caucasians comparing with Africans and Asians [5,6], and it has a peak incidence in the fifth and sixth decades of life and it is rare before 20 years of age [7]. Incidence worldwide is increasing [1].
Many risk factors have been suggested. However, many of them have not been sufficiently investigated and there are contradictory findings. Immunosuppressant drugs, familial history [8], viruses (EBV, HTLV and the herpesvirus associated with Kaposi sarcoma), congenital immunodeficiencies [1], hair dyes [9], benzene, toluene and xylene [10] were found to increase the risk of NHL. Tobacco use [11,12] and no alcohol consumption [13] were also shown to increase the risk of FL, nonetheless these findings remain controversial [14].
The natural history of the disease is associated with histologic progression in both pattern and cell type. A heterogeneous cytological composition is one of the hallmarks of FL. Usually, all follicle centre cell types are represented although in varying proportions. It should be stressed that the variation in cytological grade is a continuum, and therefore precise morphologic criteria for subclassification are difficult to establish [7].

According to the WHO criteria [15], FL are graded into 3 categories, according to the number

of centroblasts. All low-grade FLs are combined into a single category, grade 1 to 2, containing a predominance of centrocytes with less than 15 centroblasts per high-power field. Grade 3 was further subdivided into 3A (centrocytes still present) and 3B (absence of centrocytes). The proportion of centroblasts predicts clinical outcome, with a more aggressive course in cases with increased number of centroblasts. But an association between grade and clinical outcome is controversial.

Grades 1 and 2, are now, combined into one category (FL1-2 of 3) and the distinction between FL3A and FL3B is mandatory. Most patients have stage 3 or 4 disease at diagnosis, with generalized lymphadenopathy [7] with only 26% to 33% of patients presenting with stage I to II disease [16,17]. Over time, a significant fraction (10-60%) of FL evolves into an aggressive lymphoma with a diffuse large cell histology [18,19], at a rate of about 3% each year [19]. Histologic transformation is associated with very limited survival [17]. On the other hand, about 20% of cases are reported to show temporary, spontaneous regression without treatment [20]. The staging of FL is made according with the Ann Arbor Classification [3].

As previously stated the course of FL is quite variable. The two best measures of outcome at the time of diagnosis are the follicular lymphoma international prognostic index (FLIPI) and tumour grade. FLIPI includes 5 prognostic factors: age, Ann Arbor stage, haemoglobin level, extranodal involvement, and serum LDH level [21].

FLIPI (follicular lymphoma international prognostic index) has become a clinically useful prognostic tool, but gives only a rough estimate of expected outcome, as within each risk group there are marked variations in outcome. FLIPI has been devised before the era of anti CD20 monoclonal antibodies. A revised FLIPI 2 (incorporating β2 microglobulin, diameter of largest lymph node, bone marrow involvement and haemoglobin level) has been proposed [22], and, more recently, based on the mutational status of seven genes, another prognostic tool, m7-FLIPI was developed [23].

The tumour microenvironment, specifically the immunologic microenvironment comprised of T cells and dendritic cells, may influence the development and progression of FL [24]. It has been suggested that FL is an immunologically functional disease in which an interaction between the tumour cells and the microenvironment determines overall clinical behaviour [24]. Thus, there is a need for useful biomarkers for prediction of the disease course [16].

As FL is defined as a lymphoma of follicle centre B cells, it virtually always demonstrates a

growth pattern that is partially follicular [1]. Most (85%) FL [7] are associated with a t(14;18)(q32;q21) involving rearrangement of the BCL2 gene and the heavy chain of immunoglobulin gene [25]. The translocation occurs after a double-strand break at the *IGH* locus on chromosome 14 caused by a defective RAG-mediated VDJ recombination and a break at the *BCL2* locus on chromosome 18 thought to be linked to an inherent fragility at CpG sites [26]. This translocation results in constitutive expression of Bcl-2 protein and thus the inhibition of apoptosis [7]. Consequently, the cells of FL accumulate and are at risk to acquire secondary mutations, which may be associated with histologic progression. BCL-2 overexpression is virtually always the result of this translocation. Less than 10 percent of FL tumours do not harbour BCL-2 translocations and do not express BCL-2 protein. BCL-2 negativity is most commonly seen in grade 3B FL [27].

As this translocation can be detected in healthy individuals [28], this suggests that it is necessary but not sufficient for the development of FL [29] and other genetic and epigenetic lesions as well as host factors are required. The early FL progenitors then acquire secondary genetic alterations under the influence of activation-induced cytidine deaminase [30]. It is now recognized that various genetic abnormalities participate in the generation of FL, and this is assumed to constitute a major reason for the clinical heterogeneity of the disease [26].

Approximately 90 % of FL will demonstrate genetic alterations in addition to the t(14;18) [15]. Apart from the t(14;18), the most common chromosomal aberrations include non-random losses of 1p36 and 6q as well as gains of 7, 18, and X. It is also important to recognize that epigenomic alterations occur in almost every type of cancer. Recent studies have established that activation of various oncogenes and silencing of tumour suppressor genes are required for FL development and progression [31] highlighting their emerging role as a hallmark of cancer. [32]. However, FL appears to be a rather unique malignancy with epigenetic mutations occurring in nearly every patient and therefore may represent a valuable model to examine how epigenetic perturbations drive cancer in general. Mutations that modify histones are the most frequent [31].

KMT2D/MLL2 (90%) and EZH2 (25%), both histone methyltransferases, are those which are predominantly targeted by mutations, as well as the histone acetyltransferases CREBBP (30–60%), EP300 (9%), and MEF2B (15%) with almost 80% of cases having co-occurring mutations [26,33]. These somatic mutations are predominantly inactivating except those affecting EZH2 [33]. The way the EZH2 mutation promotes lymphomagenesis is the best characterized [31].

Histone methyltransferases are essential in regulating gene expression by modifying two key lysine residues: the histone 3 lysine position 4 (H3K4, KMT2D) and histone 3 lysine position 27 (H3K27, EZH2, CREBBP and EP300) marks. Promoters of actively transcribed genes are marked by the presence of a trimethyl mark on histone 3 lysine 4 (H3K4me3). By contrast, inactive genes display methylation at lysine 27 (H3K27me3) [34].

In FL it is thought that the prevailing consequence of the mutations in the histone-modifying enzymes is a shift towards aberrant repression of gene transcription by loss of active marks of transcription, catalysed by KMT2D and CREBBP/EP300, and increase in the repressive mark H3K27me3 through mutations in EZH2 [31].

The incorporation of epigenetic mutations has led to improved prognostic tools m7-FLIPI combining clinical factors with mutations in seven genes including CREBBP, ARID1A, EP300 and EZH2. EZH2 mutations were associated with good risk disease in patients with high-risk FLIPI [23], in contrast to what was found in several other tumour models where the increased expression of EZH2 is related to worse prognosis and to an increase of the capacities of invasion and tumour progression [35,36].
An epigenetic alteration that has received considerable interest is the trimethylation of H3K27, a gene silencing mark residue [37,38], catalysed by the EZH2, subunit of the Polycomb2 complex (PRC2) [39].

EZH2 mediates repression of gene transcription. It was found that EZH2 mutations in lymphoma were heterozygous, suggesting that such cancer cells were haploinsufficent for the enzymatic activity. The results could be a global deficit of H3K27 methylation and widespread derepression of gene expression [34,40].

There are conflicting findings regarding the association between EZH2 mutation and overall survival or time to transformation. While Pastore et al. [23] found that the presence of EZH2 mutations was associated with improvement in both failure-free survival and overall survival, in a different study [41] no association between EZH2 mutation and these variables was found.

Immunohistochemical staining performed on previously constructed tissue microarrays demonstrated variable expression of EZH2 and H3K27me3 in FL independent of EZH2 mutation status which supports that mechanisms other than gene mutation are affecting EZH2–H3K27me3 levels [41].

There are many opportunities to explore the mutations affecting epigenetic factors from a

therapeutic perspective. Although epigenetic therapies such as histone deacetylase inhibitors have previously been evaluated in FL, results were largely disappointing [42,43]. In parallel with these genetic discoveries, there has been a rapid development of inhibitors targeting EZH2 histone methyltransferase activity, which are demonstrating promising results in both *in vitro* and *in vivo* models. At present, three compounds have proceeded to early phase clinical trials in patients, emerging Phase I data suggest a potential role in EZH2 wild-type tumours [31].

KMT2D, also known as MLL2, codes a histone methyltransferase responsible for histone H3 lysine 4 (H3K4) trimethylation and belongs to the KMT2 family. This family of histone methyltransferases has been implicated in an extensive range of malignancies and is among the most frequent alterations in human cancer [44], its mutation represents an early event [45]. The majority of genetic alterations in this enzyme lead to truncated proteins, through decreased methylation of H3K4, resulting in the repression of gene expression [31].

Objective

The main goal of this project was to quantitatively evaluate the expression of the post-translational histone modifications H3K4me3 and H3K27me3 in a series of follicular lymphomas diagnosed and treated at Portuguese Oncology Institute of Porto, between 2007 and 2012, to determine its value as a prognostic and, possibly, predictive biomarker of therapeutic response.

Materials and Methods

This study is integrated within the scope of the PhD project of Dr. Margarida Dantas de Brito Rodrigues, entitled "Epigenética e linfomagénese – O papel das enzimas modificadoras de histonas no linfoma difuso de grandes células B e no linfoma follicular", which was approved by ethics committee (Comissão de Ética para a Saúde) of IPO Porto (CES 216/2012).

Patients and samples

Tissue biopsies from patients consecutively diagnosed with follicular lymphoma at Portuguese Oncology Institute of Porto (IPO Porto, a tertiary healthcare institution) from 2007 to 2012 were enrolled. All slides were reviewed and a representative block was selected for immunohistochemistry.

Clinical data was retrieved from patients' charts (dates of birth, diagnosis, treatment start, death; clinical stage (CS); FLIPI; treatments performed; evidence of clinical recurrence/progression; last follow-up date and vital status). Follow-up was updated as of 30th April, 2017. Patient death was rendered as FL-related (death from disease – DFD) when patients died due to disease progression or in the sequence of treatments performed, or as FL-unrelated (including either death with disease – DWD – or death with no evidence of disease – D-NED).

Immunohistochemistry and image analysis

Immunohistochemistry was performed using Novolink™ Max Polymer Detection System (Leica Biosystems, Germany). Sections (3μm-thick) were cut and microwaved for 20mins in citrate buffer at 800W for antigen retrieval. Endogenous peroxidase was blocked through incubation in hydrogen peroxide in 3% methanol for 30mins. Primary antibodies for H3K4me3 (polyclonal, clone ab8580, Abcam, Cambridge, United Kingdom) and H3K27me3 (monoclonal, clone C36B11, Cell Signaling Technology, Danvers, MA, USA) were used at 1:1000 and 1:500 dilution in 1% PBS-BSA, respectively, and incubated for 1hr and overnight at 4ºC, respectively. 3,3'-diaminobenzidine (Sigma-Aldrich™, Germany) was used for visualization and hematoxilin for nuclear counterstaining. Appropriate positive controls were used for each antibody and negative control consisted on omission of primary antibodies.

A digital image analysis system (GenASIs™, Israel) was used for H3K4me3 and H3K27me3 nuclear immunostaining quantification. Nuclei were considered positive if any staining was present, independently of intensity. Multiple fields of view were selected per slide, in order to analyse at least 5 different fields or 5000 cells. This analysis allowed the quantification of the proportion of immunostained cells, its intensity, and the Hscore (weighting between the percentage of marked cells and the intensity of the staining).

Statistical analysis

For analysis purposes, patients were divided into two Clinical Stage (CS) groups (stage I/II, stage III/IV). Cutoffs for H3K4me3 and H3K27me3 nuclear immunostaining (high vs. low) were set at 25th and 50th percentile (P25 and P50). Association between biomarkers' distribution and CS was evaluated using Chi-square test. Distribution of continuous variables between

groups was compared using non-parametric tests (Mann-Whitney or Kruskall-Wallis, as appropriate). Survival curves were constructed using Kaplan-Meier non-parametric estimator. Survival between groups was compared using Log-rank test. Statistical significance was set at $p<0.05$. Statistical analysis was performed using SPSS® Statistics for Windows, version 24.0 (SPSS, Chicago, IL, USA).

Results

Clinical and pathological characterization of the patients

Forty-eight patients with FL were included in this study. The clinical and pathological characteristics are shown in Table 1. In this sample, 27 % of patients were male, and the median age at diagnosis was 60 years. Recurrent/transformed disease was experienced by 34% of the patients.

Table 1 Clinical and pathological characteristics of the patients

		N	%
Gender	Female	35	72.9
	Male	13	27.1
Age at Diagnosis	≤60 years	26	54.2
	>60 years	22	45.8
Ann Arbor Stage	I/II	5	11.4
	III/IV	40	88.6
FLIPI	Low risk (0,1)	5	12.2
	Intermediate risk (2,3)	25	61.0
	High risk (4,5)	11	26.8

Overall survival

At the end of the study and using the date of the last consultation, the follow-up time was determined and the median was 71.5 months (range: 52.6 – 113.2 months). The overall survival was estimated as 91.3% at 2 years and 74.7% at 5 years. The disease-specific survival was estimated as 91.3% at 2 years and 77.3% at 5 years.

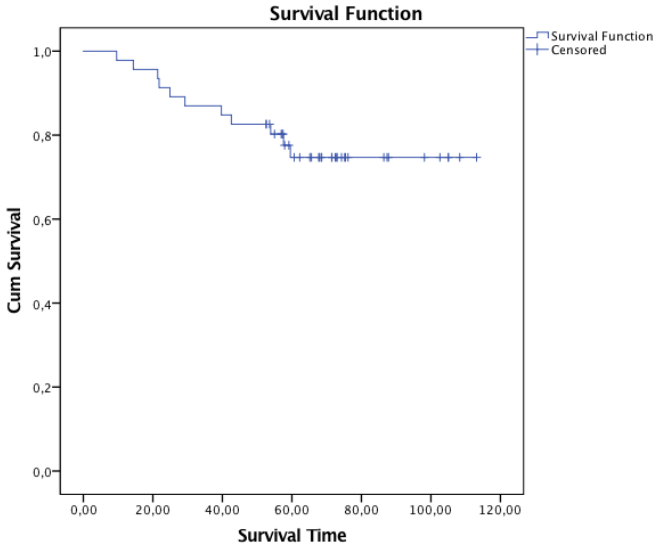

Fig. 1 Overall survival of patients with FL.

Factors that impacted on disease-specific survival

In the survival analysis, we considered the association between the disease-specific survival and the FLIPI score, Ann Arbor stage and age at the time of diagnosis. In our sample, the Ann Arbor stage was not associated with prognosis (p=0.609), whereas FLIPI score and age at the time of diagnosis were (p<0.001 and p=0.019, respectively). A lower FLIPI score and a lower age at diagnosis were associated with a better outcome. No difference in prognosis was observed concerning gender (p=0.755).

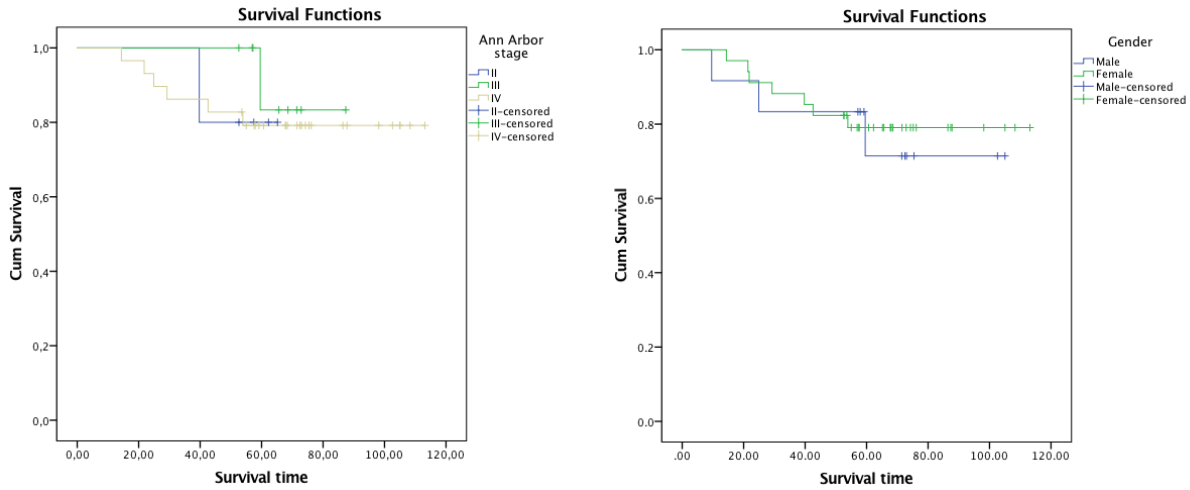

Fig. 2, 3 Analysis of disease-specific survival according to Ann Arbor stage and gender.

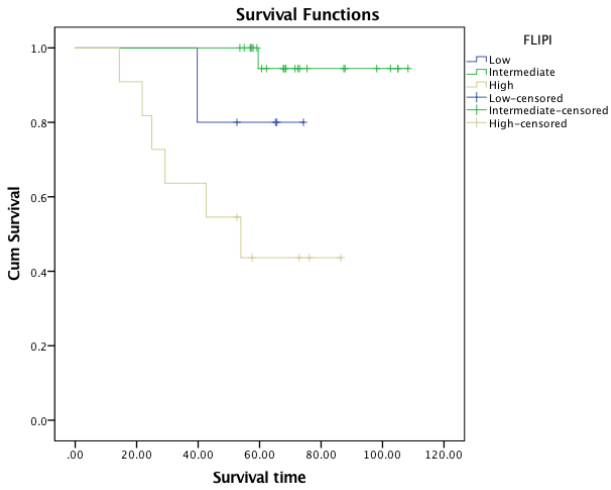

Fig. 4 Analysis of disease-specific survival according to grouped FLIPI score.

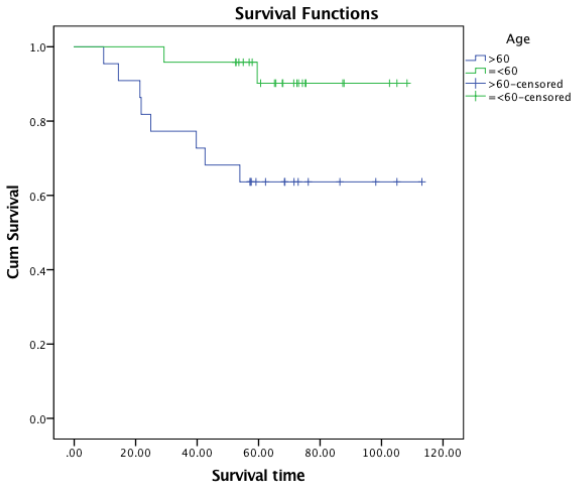

Fig. 5 Analysis of disease-specific survival according to age (>60 years, ≤60 years).

Immunohistochemistry profile:

The immunoexpression of the epigenetic markers was studied in the cells' nuclei. To evaluate the immunoexpression, the Hscore was used.

H3K4me3

In this epigenetic mark, Hscore varied between 89.20 and 300, with a median of 292.60.

In the statistical analysis of the distribution of Hscore of H3K4me3 according to gender, age,

Ann Arbor stage, transformation and FLIPI there were no statistically significant associations. We used Mann Whitney's test for all the variables except for FLIPI score, in which Kruskal Wallis' test was used. (Table 2).

Table 2 Comparison between Hscore and clinical and pathological parameters

	p
Gender	0.609
Age (≤60/>60 years)	0.543
Ann Arbor Stage	0.355
Transformation	0.762
FLIPI	0.292

The immunoexpression of H3K4me3 was not associated with the disease-specific survival using either the median (p=0.105) or the 25th percentile (p=0.179) as cutoff values.

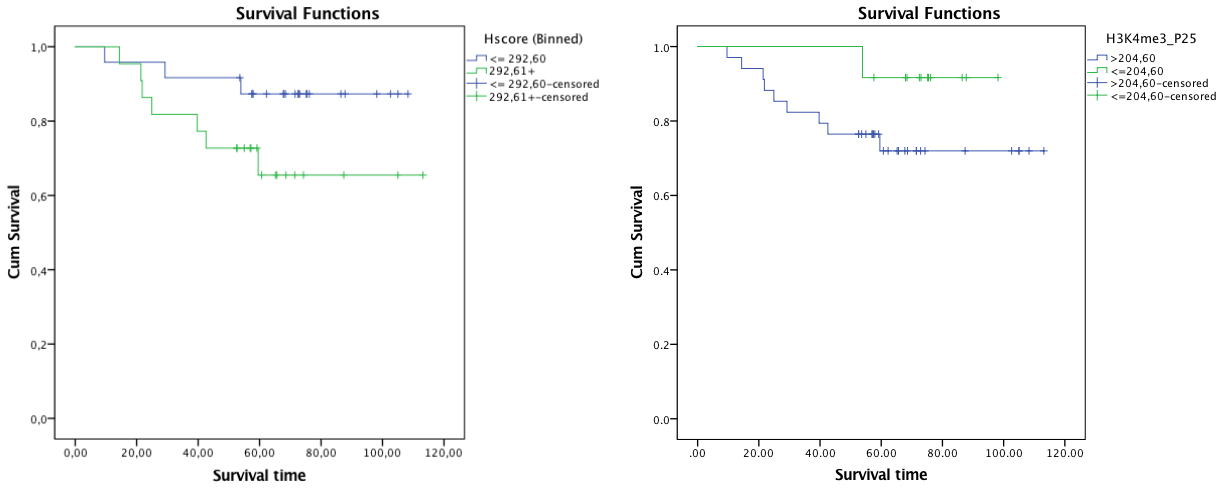

Fig. 6,7 Analysis of disease-specific survival by Hscore: in the left panel, the comparison is made using the median (292.60) and in the right panel the 25th percentile (204.60).

H3K27me3

The Hscore varied between 52.2 and 299.9, with a median of 287.65.

In the statistical analysis of the distribution of Hscore of H3K27me3 according to gender, age, Ann Arbor stage and transformation there were no statistically significant results except for FLIPI score (p = 0.038). We used Mann Whitney's test for all the variables except for FLIPI scale, in which the Kruskal Wallis' test was used. (Table 3).

Table 3 Comparison between Hscore and clinical and pathological characteristics

	p
Gender	0.862
Age (≤60/>60 years)	0.444
Ann Arbor Stage	0.644
Transformation	0.931
FLIPI	**0.038**

The immunoexpression of H3K27me3 was not associated with disease-specific survival using either the median (p=0.894) or the 25th percentile (p=0.207).

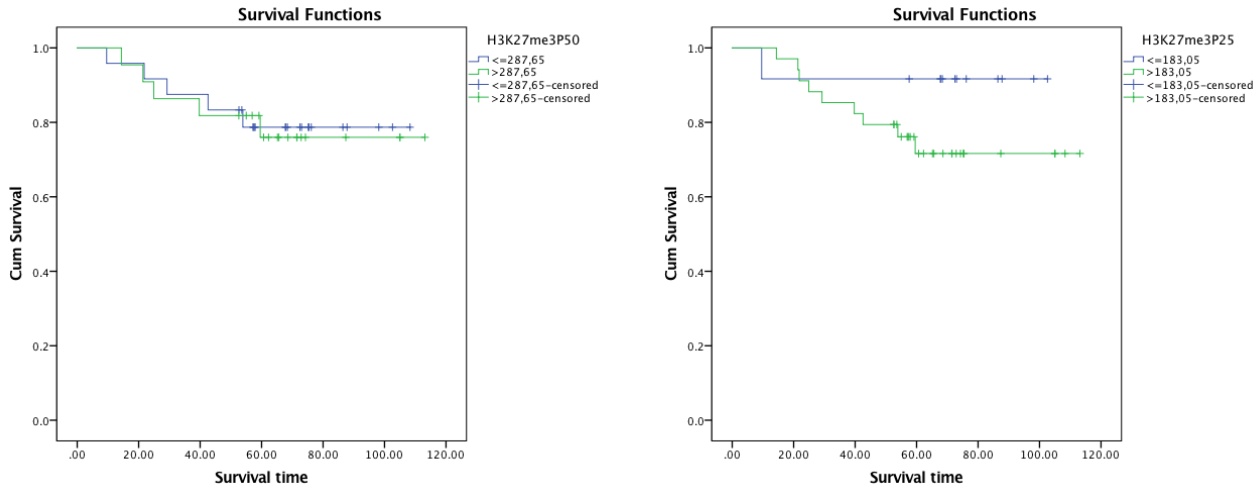

Fig. 8,9 Analysis of disease-specific survival by Hscore: in the left panel, the comparison is made using the median (287.65), and in the right panel using the 25th percentile (183.05).

Discussion

Follicular lymphoma is a clinically heterogeneous disease [16]. Although FL is most often clinically indolent, most FLs are at an advanced stage at the time of diagnosis [46]. More importantly, over time, a significant fraction evolves into an aggressive lymphoma [18,19]. Different tools have been devised to predict the prognosis of FL [21-23] and albeit these are clinically useful prognostic tool they only provide a rough estimate of the expected outcome [16]. Therefore, there is a need for useful biomarkers that more accurately predict disease course, therapy response and identifying the subset of patients at higher risk of early treatment failure and transformation [2,23].

This study was based in a series of patients diagnosed with FL and treated at IPO-Porto between 2007 and 2012. The median age (60 years) at the diagnosis is in accordance with the literature [1]. In comparison with published data [4], this series had an over-representation of women (2.70:1), also the number of patients with a clinical stage I/II was only 11% in comparison to the 26-33% usually found [16]. The proportion of patients with transformation/recurrence was 34%, well within the 10-60% previously reported [18]. In our sample, contrary to what was expected, the Ann Arbor stage was not associated with prognosis but the FLIPI score and age at the time of diagnosis were. This may mean that this series was not representative of the population diagnosed with FL.

As discussed in the previous section, two epigenetic marks were analysed: H3K4me3 and H3K27me3. In FL, it is thought that the prevailing consequence of the mutations in the histone-modifying enzymes is a shift towards aberrant repression of gene transcription. When evaluating the expression H3K4me3, it was observed that this mark was found to be expressed in variable levels but no significant association between this epigenetic mark and survival was found. Di- and trimethylation of H3K4 is linked to transcriptional activation and high levels of H3K4me3 trimethylation are associated with the promoters of actively transcribed genes [47]. A decrease of H3K4me2/me3 is observed in a range of neoplastic tissues (i.e. prostate, lung, breast and pancreatic cancer) [48,49]. In this series of FL patients we were expecting that a decrease of this marker might correlate with a worse prognosis. There are several enzymes that affect H3K4: MLL family of histone methyltransferases and LSD1 and JARID1 family of histone demethylases [47]. These enzymes have been shown to have altered activity in cancer [49]. Almost 90% of patients with FL have mutations on KMT2D [33], these mutations point to its loss of function leading to decreased methylation of H3K4, resulting in the repression of gene expression [31] and this suggests its role as a tumour suppressor gene [50,51].

LSD1 histone demethylase is responsible for the demethylation of H3K4 and H3K9, decreasing the methylation and promoting gene silencing [52]. LSD1 expression correlates with adverse outcome [49]. The levels of LSD1 are significantly increased in bladder, acute myeloid leukaemia, prostate and colorectal neoplasms [53], but no published data is available concerning altered LSD1 expression in FL. The decrease of the KMT2D activity with an increase in the activity of LSD1 could be an explanation for the decrease in methylation in this series.

When evaluating H3K27me3, which is a mark that acts as a repressor, we were expecting its increase to be correlated with a worse prognosis. However, no significant association between this epigenetic mark and survival was found. The trimethylation of lysine 27 on histone H3 (H3K27me3) is an epigenetic mark associated with transcriptional silencing by promoting a compact chromatin structure [49] and it is catalysed by EZH2 [41]. This enzyme is expressed in developing B-cells in a stage-dependent manner [34]: during early B-cell development EZH2 is required for VDJ recombination and it is subsequently downregulated in mature B-cells, but it is highly expressed again after T-cell dependent activation in germinal centre B-cells [54]. Interestingly, 25% of patients with FL were found to have an EZH2 mutation. These mutations result in a gain of function [31]. In several other tumour models the increased expression of EZH2 is related to worse prognosis and to an increase of invasion and tumour progression capacity [35,36]. Overexpression of EZH2 has been demonstrated to occur in diverse cancers, including those of the prostate, breast, kidney and ovary and may cause silencing of growth suppressive genes [35,55,49]. There are conflicting findings regarding the association between EZH2 mutation and overall survival or time to transformation. It was found that the presence of EZH2 mutations was associated with improvement in both failure-free survival and overall survival [23], in a different study [41] it did not appear to affect clinical outcome. In general, EZH2 overexpression in cancer cells seems to result in an EZH2-dependent increase in H3K27me3. However, no association was found between EZH2 and H3K27me3 expression in breast, ovarian and pancreatic cancers [56]. H3K27me3 in FL seems to be independent of EZH2 mutation status which supports that mechanisms other than gene mutation are affecting EZH2–H3K27me3 levels [41].

One explanation for the absence of significant association between EZH2 and H3K27me3 might lie in the H3K27me3 demethylases. H3K27me3 marks are removed by the JMJD3 and UTX [57]. Inactivating alterations of UTX were recently reported in a variety of tumours, particularly in multiple myeloma [58]. JMJD3 has also been linked to tumour development and

it is induced by Epstein–Barr virus and overexpressed in Hodgkin's lymphoma [59]. This suggests that both an increase or a decrease of H3K27 methylation activity may lead to malignancy, so a precise balance of this methylation is critical for normal cell growth [34]. Another group of enzymes that affects H3K27 are the histone acetyltransferases CREBBP and EP300. In FL, there is a loss of function of these enzymes resulting in inactivating genetic alterations that contribute to a repressed gene expression state through decreased acetylation of H3K27 (H3K27ac) and other target proteins [31,54].

Although some reports suggested that there was conservation of methylation profiles between pre- and post-transformation FL samples [60], which could mean that epigenetic biomarker may not be useful in predicting prognosis, with the introduction of higher resolution measurable differences in relapsed and more aggressive tumours were found [31].

There are some limitations that can be pointed out in this study. First, the size of the sample might be too small to ascertain any significant association between epigenetic marks and prognosis. Second, some characteristics of the series were not according to what would be expected, most notably the fact that there was no association between the Ann Arbor stage and prognosis and also an unbalanced number of patients with an Ann Arbor stage III/IV. Also, the evaluation of the Hscore is subject to intra- and inter observer variability, even though it was performed using GenASIs™ Software which helps reducing the variability.

In conclusion, a better understanding of the mechanism leading to B-cell lymphomas was brought by recently discovered epigenetic lesions, but more studies are needed to better clarify their role and clinical relevance. Epigenetic lesions are an opportunity for the development of improved biomarkers as well as cancer therapies.

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Annex I: Imunoexpression of H3K27me3, with respective Hscore, number of analysed cells and percentage of positive cells.

Case number	Hscore	Number of analysed cells	Percentage of positive cells
1	299.5	9311	99.8
2	297.1	6493	99.0
3	299.3	8258	99.8
4	298.6	6828	99.5
5	299.1	7708	99.7
6	296.2	8889	98.7
7	298.1	6946	99.4
8	298.1	7079	99.4
9	298.9	7294	99.6
10	153.2	4854	51.1
11	145.0	6737	48.3
12	293.2	10879	97.7
13	292.1	9017	97.4
14	298.6	7507	99.5
15	288.8	5741	96.3
16	52.2	4383	17.4
17	207.7	11475	69.2
18	293.4	9916	97.8
19	294.7	6443	98.2
20	286.5	8137	95.5
21	297.0	12897	99.0
22	299.2	7831	99.7
23	223.0	7216	74.3
24	240.4	4371	80.1
25	286.8	7491	95.6
26	266.6	5821	88.9
27	294.6	8714	98.2
28	205.0	3395	68.3
29	299.9	7687	100
30	276.0	4897	92.0
31	299.9	8365	100
32	60.9	4831	20.3
33	198.5	6695	66.2
34	33.6	5775	11.2
35	177.9	5477	59.3
36	212.3	11203	70.8
37	299.6	6178	99.9
38	255.0	5338	85.0
39	288.5	8087	96.2
40	299.9	6692	100
41	124.0	3917	41.3

42	113.8	5119	37.9
43	78.9	5197	26.3
44	152.0	5995	50.7
45	74.4	3011	24.8
46	298.6	6725	99.5
47	61.5	4811	20.5
48	265.9	5615	88.6
49	151.6	5507	50.5
50	79.0	3698	26.3
51	285.0	6656	95.0
52	251.1	4364	83.7
Control (Liver)	222.7	10465	74.2

Annex II: Imunoexpression of H3K4me3, with respective Hscore, number of analysed cells and percentage of positive cells.

Case number	Hscore	Number of analysed cells	Percentage of positive cells
1	298.4	7935	99.5
2	299.8	7467	99.9
3	299.2	9098	99.7
4	299.5	7122	99.8
5	299.3	9205	99.8
6	299.3	8757	99.8
7	299.8	10646	99.9
8	299.6	7630	99.9
9	296.0	7955	98.7
10	168.1	5625	56.0
11	233.4	5902	77.8
12	287.3	5972	95.8
13	268.8	7073	89.6
14	299.3	8837	99.8
15	291.5	10905	97.2
16	89.2	6233	29.7
17	193.5	7847	64.5
18	299.5	9103	99.8
19	299.8	8633	99.9
20	299.2	9214	99.7
21	299.3	9226	99.8
22	299.9	9311	100
23	292.5	7236	97.5
24	292.6	5615	97.6
25	299.7	8334	99.9
26	265.6	3178	88.5
27	247.1	11357	82.4
28	206.5	7202	68.8
29	298.3	7750	99.4
30	171.1	6670	57.0
31	300.0	8932	100
32	102.3	5843	34.1
33	299.2	9311	99.7
34	216.5	5800	72.2
35	155.9	5138	52.0
36	265.0	9634	88.3
37	299.7	7949	99.9
38	167.0	5874	55.7
39	255.1	6135	85.0

40	299.3	7879	99.8
41	206.5	2797	68.8
42	204.6	5911	68.2
43	192.4	7520	64.1
44	187.5	4799	62.5
45	190.2	3516	63.4
46	299.9	7993	100
47	140.9	4625	47.0
48	198.8	6410	66.3
49	189.3	6133	63.1
50	228.2	3547	76.1
51	298.5	9704	99.5
52	183.8	5476	61.3
Control (Liver)	204.4	6035	68.1

Annex III: GenASIs™ Software